Plant Physiol. (1996) 111: 487–495

Volatile Semiochemicals Released from Undamaged Cotton Leaves

A Systemic Response of Living Plants to Caterpillar Damage

Ursula S. R. Röse[1], Ara Manukian[2], Robert R. Heath, and James H. Tumlinson*

Insect Attractants, Behavior and Basic Biology Research Laboratory, United States Department of Agriculture, Agricultural Research Service, 1700 Southwest 23rd Drive, Gainesville, Florida 32604

Cotton plants (*Gossypium hirsutum* L.), attacked by herbivorous insects release volatile semiochemicals (chemical signals) that attract natural enemies of the herbivores to the damaged plants. We found chemical evidence that volatiles are released not only at the damaged site but from the entire cotton plant. The release of volatiles was detected from upper, undamaged leaves after 2 to 3 d of continuous larval damage on lower leaves of the same plant. Compounds released systemically were (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E,E*)-α-farnesene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. All systemically released compounds are known to be induced by caterpillar damage and are not released in significant amounts by undamaged plants. Other compounds, specifically indole, isomeric hexenyl butyrates, and 2-methylbutyrates, known to be released by cotton in response to caterpillar damage, were not released systemically. However, when upper, undamaged leaves of a caterpillar-damaged plant were damaged with a razor blade, they released isomeric hexenyl butyrates, 2-methylbutyrates, and large amounts of constitutive compounds in addition to the previously detected induced compounds. Control plants, damaged with a razor blade in the same way, did not release isomeric hexenyl butyrates or 2-methylbutyrates and released significantly smaller amounts of constitutive compounds. Indole was not released systemically, even after artificial damage.

Plants under herbivore attack release volatile compounds that can serve as cues that attract predators (Dicke and Sabelis, 1988; Dicke et al., 1990a, 1990b; Takabayashi et al., 1991) and parasitic wasps (Turlings et al., 1990, 1991a, 1991b; McCall et al., 1993) to the vicinity of their herbivorous prey or host. Volatiles released from plants under attack can therefore benefit both the plant, by attracting natural enemies of the herbivores that feed on its foliage, and the parasitoid, by indicating the presence of a potential host on the plant. However, volatiles released by plants immediately after the beginning of feeding damage ("fresh damage") do not differ from volatiles released by plants artificially damaged with a razor blade (Turlings et al., 1990; U.S.R. Röse, unpublished data). Early stages of plant damage are characterized by the release of "green leafy" volatiles [(Z)-3-hexenal, (Z)-3-hexenol, (Z)-3-hexenyl acetate] and some plant-specific, constitutive compounds.

Cotton (*Gossypium hirsutum* L.) plants store large amounts of monoterpenes and sesquiterpenes (Loughrin et al., 1994) in lysigenous glands (Elzen et al., 1985). These constitutive compounds, as well as the green leafy volatiles, are released immediately when the plant is damaged. Then, after several hours or on the next day of herbivore damage ("old damage"), the plants start to release herbivore-induced compounds and will continue to release these compounds for at least 3 d if damage continues (Loughrin et al., 1994). In cotton, several of these induced compounds are acyclic terpenoids [e.g. (*E*)-β-ocimene, (*E*)-β-farnesene, (*E,E*)-α-farnesene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene]. Compounds induced by insect herbivore damage also include (Z)-3-hexenyl acetate, indole, isomeric hexenyl butyrates, and 2-methylbutyrates (Loughrin et al., 1994; McCall et al., 1994).

The release of inducible terpenes [(*E*)-β-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, and (*E,E*)-α-farnesene] in cotton follows a diurnal cycle, with a peak emission of volatiles in the early afternoon (Loughrin et al., 1994). The timing of the plant signal coincides with the time of active foraging behavior of parasitic wasps (Turlings et al., 1995). For example, the highest flight activity for foraging of the braconid parasitoid *Cardiochiles nigriceps* occurred in the middle of the day in fields of cotton and beggarweed (*Desmodium purpureum*) (Snow and Burton, 1967; Lewis et al., 1972).

Several parasitoids and predators are known to exploit herbivore-induced compounds to lead them to those plants where they are likely to encounter a host or prey, as for example the generalist parasitic wasp *Cotesia marginiventris* on corn (Turlings et al., 1990), the specialist parasitic wasp *Microplitis croceipes* on cotton (McCall et al., 1993), and the predatory mite *Phytoseiulus persimilis* in lima beans (Dicke and Sabelis, 1988; Dicke and Dijkman, 1992). Since artificial damage alone does not elicit the release of significant amounts of induced compounds, those induced volatiles are a reliable indicator to the parasitoid and predator of a herbivore-damaged plant.

[1] U.S.R.R. was supported by a grant of the Gottlieb Daimler und Karl Benz-Stiftung in Ladenburg/Germany.

[2] Present address: Analytical Research Systems, P.O. Box 140218, Gainesville, FL 32614–0218.

* Corresponding author; e-mail tumlnsn@nervm.nerdc.ufl.edu; fax 1–352–374–5707.

Behavioral studies indicate that even undamaged parts of a damaged plant can be attractive to predators (Dicke and Sabelis, 1988; Dicke et al., 1990a, 1990b; Takabayashi et al., 1991) and parasitoids (Nadel and van Alphen, 1987; Turlings and Tumlinson, 1992). Those plant parts do not have to be damaged to release volatiles that attract natural enemies of herbivores.

Chemical evidence for a systemic response of corn plants to beet armyworm regurgitate, applied on artificially damaged corn seedlings, was reported by Turlings and Tumlinson (1992). Terpenoids released systemically from those corn seedlings were linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, and (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. In later experiments with corn seedlings, (*Z*)-3-hexenyl acetate, indole, α-*trans*-bergamotene, (*E*)-β-farnesene, and (*E*)-nerolidol were also found to be released systemically (Turlings, 1994). The release of these compounds is known to be induced by herbivore-feeding damage on corn.

Since herbivore-induced emissions of plant volatiles appear to play a very important role in the foraging behavior of parasitoids and predators, we conducted experiments with living cotton plants to investigate the volatiles released from undamaged parts of a caterpillar-damaged plant. As far as we know, no previous study has shown chemical evidence for a systemic release of caterpillar-induced volatiles from intact, living plants. To determine the release pattern of cotton volatiles from undamaged leaves in response to caterpillar damage on the lower leaves of the plant, we monitored the volatile emission over a period of 4 d. Our experimental design excludes any possibility of adsorption of volatiles from the damaged site by the undamaged leaves of the injured plant.

MATERIALS AND METHODS

Plants

Approximately 6-week-old cotton plants (*Gossypium hirsutum* L. [Malvaceae], var Deltapine acala 90) with eight fully developed leaves in addition to the two cotyledons were used in all experiments. Cotton was reared in a greenhouse in a potting soil:vermiculite mixture (3:1) with natural light, under Florida summer conditions (14 h:10 h light:dark photoperiod, 85 ± 10% RH, and 35± 10°C). Each cotton seedling was planted in a 16-cm-diameter pot.

Insects

Beet armyworm (*Spodoptera exigua*, Hübner [Noctuidae]) larvae were obtained from the U.S. Department of Agriculture rearing facilities in Gainesville, FL. They were reared on an artificial diet, based on pinto beans, according to the method of King and Leppla (1984). To encourage immediate feeding after being placed on the plants, third-instar larvae were starved for 24 h prior to all experiments.

Volatile Collection from Undamaged Leaves

Volatile chemicals were collected daily from living cotton plants over 4 consecutive d in the greenhouse. Each day two continuous 3-h volatile samples were taken at times when partially damaged plants emitted a maximum of volatiles (12–3 and 3–6 PM; Loughrin et al., 1994). Four beet armyworm larvae were allowed to feed only on the lower four leaves of a cotton plant, while volatiles were collected from all four undamaged upper leaves. Volatiles were also collected simultaneously from the upper leaves of an undamaged control plant under identical conditions (Fig. 1).

To collect volatiles from the undamaged parts of a caterpillar-damaged plant (systemic treatment; designated SYST) and a control (designated CTRL) plant, the upper four leaves of each plant were enclosed in separate "guillotine" volatile collection chambers that were part of an automated volatile collection system previously described (Manukian and Heath, 1993; Heath and Manukian, 1994). Briefly, the guillotine collection assembly consisted of an air inlet diffuser cap containing multiple layers of a compressed charcoal-infused medium used for air purification and laminar flow (Heath and Manukian, 1992) and a large glass chamber (400-mm-long × 152-mm-o.d. × 4-mm-thick Pyrex glass) on top of a multiport guillotine base. The multiport guillotine base contained concentric gas-sampling ports and two Teflon-coated removable blades that closed off the collection chamber in a guillotine-like fashion around the stem of the plant, leaving only a small opening for the stem where the blades were fitted together. This system allows for the collection of volatiles from the upper portion of the cotton plants while completely isolating the lower section of the plant where caterpillars are feeding in the systemic treatment (Fig. 1).

Purified air entered the system through the air diffuser inlet on top of the glass chamber at a controlled rate of 5 L/min. Volatile collector traps (150 mm long × 5 mm o.d.), containing 50 mg of Super-Q (catalog no. p/n 2735; Altech Associates, Deerfield, IL) as an adsorbent, were inserted in the side sampling ports located symmetrically around the base of the multiport guillotine base. Volatiles emitted from the upper portion of the cotton plant enclosed within the glass chamber were swept downward by the incoming pure laminar air flow. They were sampled at the bottom of the chamber by pulling air at a rate of 1 L/min through the volatile collection traps from a controlled vacuum source attached to each volatile collector trap from the automated volatile collection system. Thus, 20% of the air passed through the collector traps during the 3-h collection period. The remaining 80% of excess air escaped through the opening at bottom of the guillotine around the stem of the plant, which was loosely plugged with cotton balls to prevent any abrasion of the plant stem on the guillotine blades. This positive-pressure venting provided a barrier against all ambient air and prevented volatiles from the lower, damaged part of the plant from entering into the collection chamber containing the upper, undamaged part of the plant (Fig. 1).

For the systemic treatment, two caterpillars per leaf were enclosed on the lower leaves of the plant, in a cage consisting of halves of a Petri dish. A circle was cut out of both sides of the Petri dish and covered with gauze to allow ventilation. A Styrofoam ring cushioned each side of the

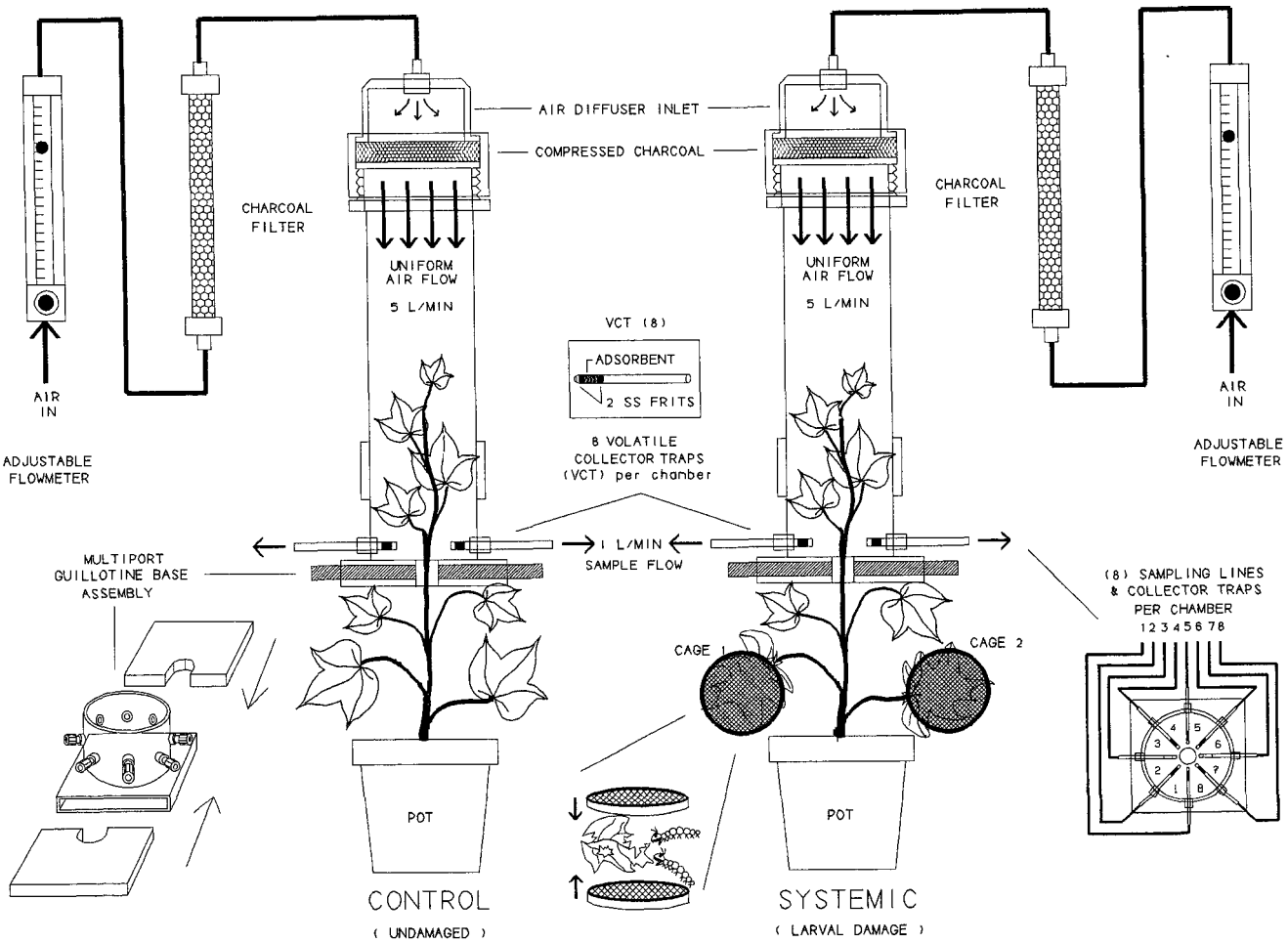

Figure 1. Apparatus used to collect volatiles from the undamaged, upper leaves of a systemically induced cotton plant (SYSTEMIC) and undamaged control leaves (CONTROL). Clean, humidified air entered the glass collection chamber through the top and swept over the upper leaves enclosed in the glass chamber, carrying the released volatiles downward. Twenty percent of the volatiles were pulled through one of the collector traps containing Super Q adsorbent. Excess air escaped through the guillotine-like bottom of the chamber, around the stem of the cotton plant, preventing air from entering the collection system. Beet armyworm larvae were caged around two of the lower leaves. Each cage consisted of halves of a modified Petri dish that were clamped together. SS, Stainless steel.

cage that touched the leaf to prevent abrasion of the leaf. The cage was clamped together with curl clips and supported by bamboo sticks. A total of four beet armyworms were placed on the lower leaves of the cotton plant on d 1 at 11 AM and were replaced with new, starved larvae every day for 5 d. On d 3 both cages were moved to new leaves to avoid the complete consumption of the leaf. In control experiments, empty cages were clamped on the bottom leaves of control plants for 5 d and volatiles were collected from the top leaves.

Volatile Collection from Artificially Damaged Leaves

On d 5 the top parts of the systemic treatment and the control plants, each with four leaves, previously used for volatile collection were cut off at the stem, and then these leaves were artificially damaged with a razor blade. Immediately, the artificially damaged systemic treatment (ART-SYST) and the artificially damaged control (ART-CTRL) leaves were placed in separate volatile collection chambers in the laboratory and volatiles were collected from 12 to 3 PM.

The laboratory volatile collection system modified from that of Turlings et al. (1991b) consisted of two parallel glass chambers, each made of two separate parts. The first part of each glass chamber consisted of a 5-cm-long, 0.5-cm-o.d. inlet, which widened into a section (10-cm-long section, 3 cm o.d.) containing a glass frit to assure laminar air flow. The second part, containing the plant sample, was 15 cm long (3.8 cm o.d.) with a 5-cm-long (0.5 cm o.d.) outlet. Both parts of each glass chamber had fitting glass ball joints that were clamped together. A volatile collection trap (6 cm long, 0.5 cm o.d.) with 25 mg of Super-Q adsorbent was connected to the 0.5-cm outlet with a brass Swagelok fitting (Crawford

Fitting Co., Niagara Falls, Ontario, Canada) containing Teflon ferules. To collect volatiles with the push-pull system, humidified air purified with an activated charcoal filter was blown through the glass chambers and exited through a volatile collection trap. An equal airflow of 600 mL/min through each chamber was controlled by flowmeters (Aalborg Instruments, Monsey, NY) downstream of the collection filters and connected to a vacuum.

Sample Analysis

Volatiles were extracted from the collector traps by washing with 170 μL of methylene chloride (capillary GC/GC-MS solvent, Burdick and Jackson, Muskegon, MI) for traps containing 25 mg of adsorbent and 200 μL for traps containing 50 mg of adsorbent. Internal standards were added (600 ng each of *n*-octane and nonyl acetate in 60 μL of methylene chloride) to the extract. Of each collection sample, 1 μL was analyzed on a Hewlett-Packard model 5890 gas chromatograph. Samples were injected by a Hewlett-Packard auto injector model 7673 in on-column mode. The gas chromatograph was equipped with an on-column capillary injector system and flame ionization detector. Data collection, storage, and subsequent analysis were performed on a Perkin Elmer chromatographic data system. Helium at a linear flow velocity of 19 cm/s was used as a carrier gas. All samples were analyzed on a fused silica capillary column (Quadrex Corporation, New Haven, CT), 50 m × 0.25 mm i.d., with a 0.25-μm-thick film of bonded methyl silicone. The temperature of the column oven was maintained at 50°C for 3 min and then increased at a rate of 7°C/min to a final temperature of 190°C and maintained at 190°C for 10 min. The injector temperature was tracked by the oven temperature and set 3°C higher than the oven temperature. The detector temperature was set at 275°C. For better separation, volatiles collected from artificially damaged leaves were also analyzed on a DB5MS column (J&W Scientific, Folsom, CA), 30 m × 0.25 mm i.d., with a 0.1-μm-thick film of bonded methyl silicone with 5% phenyl. The column oven was maintained at 40°C for 3 min and then programmed at 5°C/min to 220°C, which was maintained for 10 min. The injector and detector temperatures were set as described above.

To identify compounds, volatiles were analyzed by GC-MS with a Finnigan (San Jose, CA) ITS-40 Magnum (ion-trap) mass spectrometer operated in electron impact and chemical ionization modes. For GC-MS the same DB5MS was used with helium as a carrier gas, and for chemical ionization isobutane was used as the reagent gas. Constituents of the plant volatile emission were identified by comparison of mass spectra with spectra in the Environmental Protection Agency-National Institutes of Health database, the Environmental Protection Agency-National Institute of Standards and Technology database, and spectra obtained of authentic compounds. Also GC retention times of plant volatiles were compared with GC retention times of those authentic compounds on the methyl silicone column and the DB5MS column.

Statistical Analysis

Since the amounts of the various volatiles released per plant frequently decreased below detectable limits, the assumption that these amounts are normally distributed is unreasonable. Therefore, changes in amounts of volatiles released per plant over time and differences between SYST and CTRL (or ART-SYST and ART-CTRL) amounts of volatiles released per plant were analyzed nonparametrically. The Mann-Whitney *U* test was used to determine the significance of daily differences in volatile amounts between five SYST and five CTRL leaf replicates and also between the five ART-SYST and five ART-CTRL replicates. An exact form of the Friedman rank sum test with adjustment for pairwise multiple comparisons (Hollander and Wolfe, 1973) was used to determine separately the significance of changes in SYST and CTRL volatile amounts released on d 2, 3, and 4 relative to the amounts released on d 1. Comparisons yielding a P value ≤ 0.05 were considered to be statistically significant. In keeping with the nonparametric analytic approach to the data, observed volatile amounts were summarized by the median and corresponding range (minimum-maximum) for each treatment condition.

RESULTS AND DISCUSSION

Systemic Volatile Release

Volatiles released systemically from the cotton plants from 12 to 3 PM were not significantly different from those collected from 3 to 6 PM. Therefore, we present only data from collections from 12 to 3 PM in Table I.

During the first hours of feeding damage on d 1, the undamaged leaves of both the treated and the control plants released only small amounts of volatiles (Fig. 2, d 1; Table I). On d 2, after continuous feeding damage by beet armyworm larvae on the lower leaves for 24 h, (*Z*)-3-hexenyl acetate, linalool, and (*E*)-4,8-dimethyl-1,3,7-nonatriene were released in significantly higher amounts from the undamaged leaves of the caterpillar-damaged plant (SYST) compared to the undamaged control (CTRL) leaves (Table I; Fig. 2, d 2). The range of the amounts of compounds detected on d 2 varied between the plants from each replicate. Although SYST leaves from most plants did not release (*E*)-β-farnesene and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene on d 2 (Table I, d 2, medians), some plants appeared to respond faster (Table I, d 2, maxima). CTRL leaves released (*Z*)-3-hexenyl acetate and (*E*)-4,8-dimethyl-1,3,7-nonatriene, in addition to small amounts of other compounds (Table I), but no (*E*)-β-farnesene or (*E*,*E*)-α-farnesene was detected. On d 3 significantly more (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, and (*E*,*E*)-α-farnesene were released systemically from the SYST leaves compared to the CTRL leaves (Fig. 3). (*Z*)-3-hexenyl acetate was still released in large amounts from the SYST leaves compared to CTRL leaves on d 3, but the difference was not statistically significant ($P \leq 0.09$). (*E*)-β-farnesene and (*E*,*E*)-α-farnesene were not released in detectable amounts from CTRL leaves, whereas (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatet-

Table I. *Composition of volatile blends collected between 12 and 3 PM (medians over five replications with range of values [minimum to maximum] shown in parentheses[a] from the undamaged systemic treatment (SYST) and undamaged control (CTRL) for 4 d*

n, Compound not detectable.

Peak	Compound	Nanograms of Compound Emitted over 3 h							
		d 1		d 2		d 3		d 4	
		SYST	CTRL	SYST	CTRL	SYST	CTRL	SYST	CTRL
1	(*Z*)-3-hexenyl acetate	42 (n–675)	72 (n–223)	528[b] (137–1494)	101 (n–570)	552 (n–3100)	73 (n–399)	1204[b] (74–2065)	n (n–126)
2	(*E*)-β-ocimene	n (n–71)	48 (n–88)	335 (n–756)	n (n–160)	1099[b,c] (449–1784)	60 (n–201)	1021[b,c] (608–1981)	n (n–204)
3	Linalool	n (n–54)	n (n–55)	571[b] (100–763)	n (n–148)	1058[b,c] (362–1604)	223[d] (75–892)	638[b,c] (377–1142)	193 (n–240)
4	(*E*)-4,8-dimethyl-1,3,7-nonatriene	n (n–22)	79 (n–147)	1203[b] (242–1549)	49 (n–340)	2204[b,c] (1008–4274)	645[d] (428–1489)	1653[b,c] (1089–3661)	633 (n–1544)
5	(*E*)-β-farnesene	n (n–n)	n (n–n)	n (n–210)	n (n–n)	2493[b,c] (97–2951)	n (n–n)	2091[b,c] (1500–4792)	n (n–n)
6	(*E,E*)-α-farnesene	n (n–52)	n (n–n)	118 (n–620)	n (n–n)	289[b,c] (221–2552)	n (n–n)	1136[b,c] (447–2805)	n (n–n)
7	(*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	n (n–47)	n (n–n)	n (n–849)	n (n–64)	1084[c] (135–2604)	959[d] (231–3261)	387 (n–625)	786 (n–1105)

[a] In keeping with the nonparametric analytic approach to the data, observed volatile amounts were summarized by the median and corresponding range (minimum-maximum) for each treatment. [b] The Mann-Whitney *U* test was used to determine the significance of daily differences in volatile amounts between five SYST and five CTRL leaf replicates. These comparisons yielded a P value ≤ 0.05 and were considered to be statistically significant. [c,d] An exact form of the Friedman rank sum test with adjustment for pairwise multiple comparisons was used to determine separately the significance of changes in SYST and CTRL volatile amounts released on d 2, 3, and 4 relative to the amounts released on d 1; significant differences ($P \leq 0.05$) between SYST leaves on d 2, 3, and 4 from d 1 are indicated by the symbol [c], and significant differences between CTRL leaves on d 2, 3, 4 from d 1 are indicated by the symbol [d].

raene was released in large amounts from both plants (Table I).

No additional compounds were detected on d 4. Even in experiments that continued over 10 d, no additional compounds were detected (data not shown). The SYST leaves released significantly more (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, and (*E,E*)-α-farnesene on d 4 than the CTRL leaves. (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene was released from both plants in comparable amounts.

SYST leaves released compounds that were not detected from CTRL leaves or only in significantly smaller amounts, clearly indicating that the plant was responding systemically on d 3. Adsorption of volatiles released by the caterpillar-damaged leaves outside the collection system by the undamaged leaves inside the collection system was highly unlikely because positive air pressure within the system prevented air from entering the volatile collection chamber (Fig. 1).

A significant ($P \leq 0.05$) increase in the amounts of volatiles released from SYST leaves over the time course of 4 d was observed for the amounts of systemically released (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E,E*)-α-farnesene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene on d 3 when compared to d 1 (Table I). The release of (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene was significantly higher only on d 3 compared to d 1 but declined on d 4 (Table I). Although the amount of (*Z*)-3-hexenyl acetate appeared to increase over the 4 d (Table I), the differences were not significant.

Over the time course of 4 d the CTRL leaves released significantly more linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene only on d 3 compared to d 1, but the release decreased significantly on d 4.

The same release pattern of (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene over 4 d was observed for volatiles collected from SYST and CTRL leaves. The amount released from both treatments on d 3 was significantly higher than the amount released on d 1, with a significant decrease on d 4. In both treatments the amount of (*Z*)-3-hexenyl acetate did not change significantly over time.

Previously in our laboratory, volatiles were collected from caterpillar-damaged cotton plants (Loughrin et al., 1994, 1995; McCall et al., 1994). These studies showed that caterpillar feeding induced, among other compounds, the release of isomeric hexenyl butyrates and 2-methylbutyrates and indole from damaged leaves. All of the compounds collected from SYST leaves in our experiment were inducible compounds, but no isomeric hexenyl butyrate or 2-methylbutyrates or indole were detected. This may indicate a difference between the induced compounds released from the damaged site itself and the induced compounds released systemically from undamaged leaves.

Some of the compounds reported here [linalool, (*E*)-β-farnesene, and (*E,E*)-α-farnesene] were not found when volatiles were collected prior to peak emission times by McCall et al. (1994) and Loughrin et al. (1995). Loughrin et al. (1994) showed that these and other induced compounds

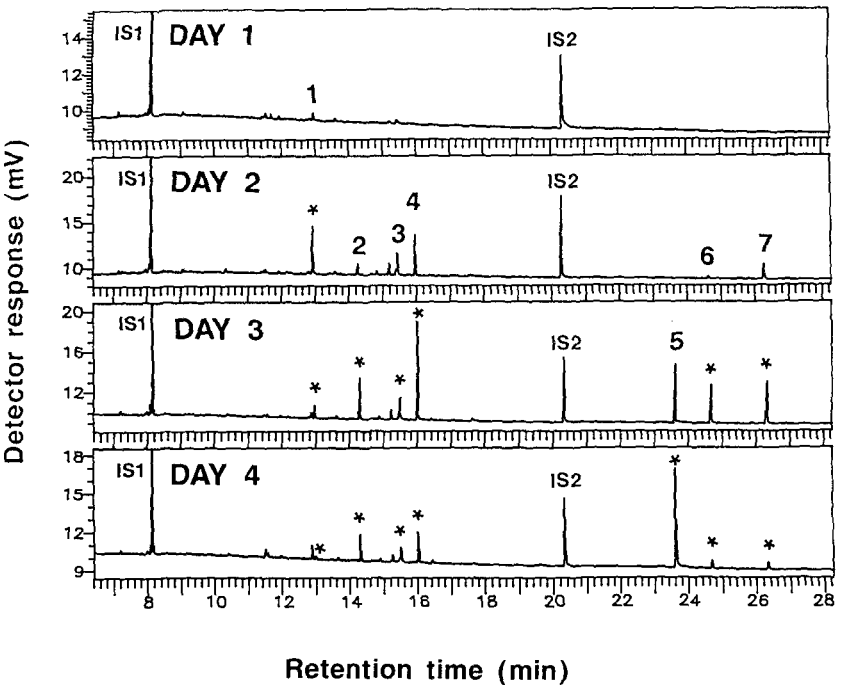

Figure 2. Chromatographic profiles after analyses on the methyl silicone capillary column of systemically released volatiles from the upper, undamaged leaves of a beet armyworm-damaged cotton plant collected from 12 to 3 PM each day for 4 d. Compounds: 1, (*Z*)-3-hexenyl acetate; 2, (*E*)-β-ocimene; 3, linalool; 4, (*E*)-4,8-dimethyl-1,3,7-nonatriene; 5, (*E*)-β-farnesene; 6, (*E,E*)-α-farnesene; 7, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Added reference compounds were *n*-octane (IS1) and nonyl acetate (IS2). Peak numbers are the same as in Table I. An asterisk (*) indicates a compound in one of the lower chromatograms that aligns with a peak in a chromatogram above it.

collected from a beet armyworm-damaged cotton plant follow a diurnal rhythm with a peak emission from 12 to 3 3 to 6 PM. The importance of the diurnal cycle for the volatile emission of induced compounds was also shown for corn (Turlings et al., 1995), and the peak emission times may vary somewhat for different plant species. In addition, the developmental stage of the plant may play a major role, as shown for volatiles released by cucumber leaves of different ages (Takabayashi et al., 1994), and in some cases the developmental stage of the attacking herbivore (Takabayashi et al., 1995). We have not collected systemically released volatiles over 24 h, but it is conceivable that their release may also follow a diurnal rhythm.

A significant increase, compared to d 1, in the release of (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E,E*)-α-farnesene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene from SYST leaves did not occur until d 3. However, when volatiles were collected

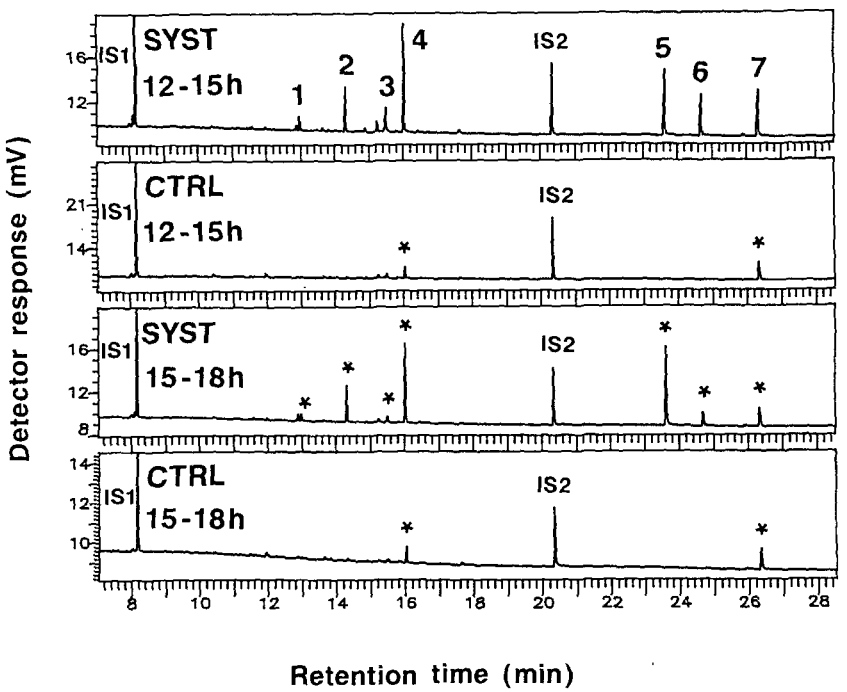

Figure 3. Typical chromatographic profiles after analyses on the methyl silicone capillary column of volatiles released from the systemic treatment (SYST) and control (CTRL) leaves on d 3 at two collection times (12-3 and 3-6 PM). Peak numbers are the same as in Figure 2 and Table I. An asterisk (*) indicates a compound in one of the lower chromatograms that aligns with a peak in a chromatogram above it.

from the entire caterpillar-damaged plant, these compounds were detected in significantly higher amounts after only 2 d (Loughrin et al., 1994). SYST leaves released significantly more (Z)-3-hexenyl acetate, linalool, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene on d 2 when compared to CTRL leaves. This may indicate that the systemic release of induced compounds that required the transport of a signal in the plant was slower than the release of induced volatile compounds from a caterpillar-damaged site. Furthermore, the induction of a systemic release of volatiles in cotton after beet armyworm damage took longer than the induction of a systemic release of volatiles in corn seedlings after the application of caterpillar regurgitate to artificially damaged corn leaves. Whereas corn seedlings expressed a systemic response after only 5 to 6 h (Turlings and Tumlinson, 1992), in our experiments cotton plants did not express a clear systemic response until after about 48 h. The slower-growing, perennial cotton plants contain high amounts of constitutive terpenoids (Elzen et al., 1985) compared to corn (Turlings et al., 1990) and may partially rely on those compounds for their immediate defense. Therefore the systemic response may only be induced by higher amounts or longer periods of damage, thus minimizing the cost of defense.

A change in volatile release over time in the CTRL leaves indicated that the control plants were also subject to stress. Empty cages that were clamped on the bottom leaves of some control plants over 5 d showed no effect on the release of volatiles from the top leaves when compared to the release of volatiles from plants without cages. Plant stress causing volatile release could have been due to higher temperature (Sharkey and Singsaas, 1995) or lower RH in the glass collection chamber. The similarity in the amounts of (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene released by CTRL and SYST leaves may indicate a general stress response of the plant rather than a specific response to caterpillar damage. However, the lower leaves of the control plant and the damaged plant shared the same air space in close vicinity. Damaged cotton leaves release high amounts of terpenoids (Loughrin et al., 1994; McCall et al., 1994), and an effect of those volatiles on the lower leaves of the control plant cannot be excluded as a possible induction factor. Possible plant-plant communication has been suggested for cotton plants (Bruin and Sabelis, 1989; Bruin et al., 1995) and some tree species (Baldwin and Schultz, 1983).

Release of Volatiles after Artificial Damage of SYST Leaves

The systemically released compounds did not include all of the induced compounds previously reported from caterpillar-damaged plants (Loughrin et al., 1994). We conducted experiments to investigate whether the missing induced compounds, indole and the isomeric hexenyl butyrates and 2-methylbutyrates, would be released after artificial damage of the SYST leaves. As expected, most of the compounds released after artificial damage of the upper leaves of treated and control plants were green leafy volatiles and constitutive terpenoid compounds such as α-pinene, β-pinene, myrcene, and caryophyllene that are typical for freshly damaged cotton leaves (McCall et al., 1994). Those volatiles were released in addition to the induced compounds previously detected in SYST leaves. Several compounds were released from the artificially damaged upper leaves of the systemic treatment (ART-SYST) in significantly larger amounts than from artificially damaged control leaves (ART-CTRL) (Table II). This is not only the case for the induced compounds but also for most of the constitutive compounds. Because care was taken to damage upper leaves of both plants to the same extent, and because no significant differences could be found for green leafy volatiles between the two treatments (Table II, peaks 1-3), it is likely that the differences in constitutive compound release are also induced by caterpillar feeding. In addition to the volatiles released by artificially damaged upper leaves of both plants, ART-SYST leaves released isomeric hexenyl butyrates and 2-methylbutyrates not detected from SYST leaves, which indicates that leaf damage was necessary for their release (Table II).

The absence of significant amounts of indole in the volatile blend released from SYST and ART-SYST leaves compared to volatiles released from a whole, partially damaged plant indicates a difference in the volatile induction. Indole is released in large amounts from cotton leaves damaged for several hours by caterpillars (Loughrin et al., 1995; McCall et al., 1994) but is not detected in significant amounts from leaves that are only artificially damaged with a razor blade (U.S.R. Röse, unpublished data). Thus, in cotton only the caterpillar-damaged site itself appeared to release indole. This finding could be due to microbial activity on the damaged leaves or due to a difference in the induction at the damaged site compared to that involving a signal that travels in the plant and induces the systemic release of volatiles in the upper leaves. However, corn seedlings that were artificially damaged and treated with beet armyworm regurgitate released indole systemically (Turlings, 1994).

Differences in the Amount of Induced Compounds Released from SYST and ART-SYST Leaves

The amounts of the induced compounds (Z)-3-hexenyl acetate and (*E*)-β-ocimene released from ART-SYST leaves on d 5 (Table II) were significantly higher than from SYST leaves on d 4 (Table I). However, the release of other induced compounds, linalool, (*E*)-β-farnesene, and (*E*,*E*)-α-farnesene, was lower from ART-SYST leaves (Table II) when compared to SYST leaves (Table I). Still, the amount of linalool and (*E*)-β-farnesene released from ART-SYST leaves was higher than the amount released from ART-CTRL leaves (Table II). No difference in the released amounts of the induced compounds (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene was observed, whether the systemically treated plant was artificially damaged or not.

The higher release of the induced compounds (Z)-3-hexenyl acetate and (*E*)-β-ocimene and the increased amount of constitutive terpenoids released from ART-SYST leaves compared to the amount released from SYST leaves may indicate that these compounds are synthesized in greater amount in SYST leaves and stored until the plant is attacked again. A resulting burst of induced and constitutive volatiles after damage may increase the attractiveness of the plant to para-

Table II. *Composition of volatile blends collected between 1200 and 3 PM (medians over five replications with range of values [minimum to maximum] shown in parentheses[a] from the artificially damaged systemic treatment (ART-SYST) and the artificially damaged control (ART-CTRL) on d 5*

n, Compound not detectable.

Peak No.	Compound	Nanograms of Compound Emitted over 3 h	
		ART-CTRL	ART-SYST
1	(*Z*)-3-hexenal	1,034 (955–3,189)	1,597 (501–1,813)
2	(*E*)-2-hexenal	1,577 (914–3,116)	2,066 (1,431–7,430)
3	(*Z*)-3-hexenol	9,922 (6,731–57,901)	16,981 (5,783–18,985)
4	Unidentified	1,403[b] (929–2,740)	4,079 (2,534–6,817)
5	Unidentified	786 (642–4,986)	1,887 (1,068–3,039)
6	Unidentified	307[b] (144–594)	1,884 (1,031–2,244)
7	α-Pinene	6,510[b] (5,407–12,651)	16,378 (13,680–36,069)
8	Camphene	110[b] (70–261)	255 (149–424)
9	β-Pinene	1,171[b] (971–2,024)	3,172 (2,634–7,167)
10	Myrcene	2,269[b] (967–4,729)	6,860 (5,521–17,649)
11	(*Z*)-3-hexenyl acetate	13,302[b] (11,091–66,303)	86,907 (47,193–155,600)
12	Hexyl acetate	467[b] (196–1,812)	3,882 (2,613–8,340)
13	(*E*)-2-hexenyl acetate	193[b] (39–286)	4,567 (1,555–11,812)
14	Limonene	659[b] (481–1,298)	1,729 (1,157–3,097)
15	β-Ocimene	107[b] (28–423)	3,878 (2,610–7,374)
16	Linalool	18[b] (n–54)	188 (122–427)
17	(*E*)-4,8-dimethyl-1,3,7-nonatriene	49[b] (28–154)	709 (204–1,570)
18	(*Z*)-3-hexenyl butyrate	n[b] (n–37)	1,326 (602–1,777)
19	(*E*)-2-hexenyl butyrate	n[b] (n–33)	886 (141–1,278)
20	(*Z*)-3-hexenyl-2-methyl-butyrate	n[b] (n–14)	246 (72–1,639)
21	(*E*)-2-hexenyl-2-methyl-butyrate	n (n–17)	262 (n–1,534)
22	(*Z*)-Jasmone	n (n–5)	54 (n–57)
23	β-Caryophyllene	2,312[b] (1,193–7,570)	9,913 (7,870–13,990)
24	α-Bergamotene	80[b] (47–156)	242 (152–465)
25	α-Humulene	651[b] (316–2,030)	2,614 (2,065–3,715)
26	(*E*)-β-Farnesene	43[b] (n–97)	1,077 (704–1,704)
27	Unknown sesquiterpene hydrocarbon	146[b] (83–255)	441 (294–955)
28	(*E,E*)-α-Farnesene	n (n–16)	45 (n–830)
29	γ-Bisabolene[c]	1,214[b] (777–2,309)	4,143 (2,982–9,104)
30	(*E,E*)-4,8,12-trimethyl-1,3,7,11-trideca-tetraene	103 (37–334)	168 (30–294)
31	β-Bisabolol (tentative)[c]	946 (331–1,384)	1,462 (864–2,999)

[a] In keeping with the nonparametric analytic approach to the data, observed volatile amounts were summarized by the median and corresponding range (minimum-maximum) for each treatment. [b] The Mann-Whitney *U* test was used to determine the significance of differences in volatile amounts of each compound between five ART-SYST and five ART-CTRL leaf replicates on d 5. These comparisons yielded a P value ≤ 0.05 and were considered to be statistically significant. [c] Tentative identification based on comparison of mass spectra with spectra of the National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health data base; authentic standard not available for direct comparison.

sitoids. However, plant volatiles may also attract plant pests such as the boll weevil (Gueldner et al., 1970). Mated female moths of cabbage loopers (*Trichoplusia ni*) are attracted to cotton plants damaged by caterpillars for about 20 h, whereas the attractiveness of cabbage plants to the moth is decreased after damage (Landolt, 1993). It is interesting that for oviposition these moths preferred undamaged cotton and cabbage plants. The systemic release of compounds throughout the entire cotton plant after 2 d may decrease the attractiveness of the plant to the moth for oviposition even more. Several sesquiterpenes found in pigment glands of cotton plants are toxic to cotton insect pests and exhibit feeding-deterrent activity toward cotton pests (Meisner et al., 1977). Therefore, SYST leaves with elevated levels of constitutive terpenoids are likely to be less preferred or unsuitable as caterpillar food. Higher amounts of constitutive compounds in systemically induced leaves could function as a feeding deterrent for herbivores and protect the youngest leaves that are more attractive to herbivores than older leaves because of their high concentrations in nutrients (Mattson, 1980; Rauppe and Denno, 1983). Beet armyworm larvae that are given a choice of systemically induced cotton leaves and undamaged control leaves avoided feeding on the induced leaves after 3 d but fed on induced leaves for the first 2 d (Alborn et al., 1996). Therefore, the production of feeding-deterrent compounds coincides with the release of systemically induced volatiles in cotton plants, possibly indicating to the moth that a plant is unsuitable for oviposition. The systemically released (*E*)-β-farnesene, a known alarm pheromone in aphids (Bowers et al., 1972), may even protect induced cotton plants from aphid infestation.

The ability of crop plants to react faster to caterpillar damage and to release higher amounts of induced defense chemicals should be considered by plant breeders as criteria in

selecting improved cultivars. A Florida naturalized cotton variety is reported to have higher contents of constitutive terpenoids and is a less preferred food source for Lepidoptera larvae (Loughrin et al., 1995). With the help of genetic techniques it may be possible to engineer plants that combine the high yield of cultivated varieties with the greater ability of naturalized varieties to defend themselves.

ACKNOWLEDGMENTS

We thank A.T. Proveaux and P.M. Brennan for technical assistance, P. Kubilis for statistical advice, J. Lockerman for assistance, V. Wenzel for support and encouragement, and the Delta and Pine Land Company (Hollandala, MS) for donation of the seeds. We are grateful to Ted Turlings, John Loughrin, and Peter Landolt for their helpful comments concerning the initial manuscript.

Received December 8, 1995; accepted March 14, 1996.

LITERATURE CITED

Alborn HT, Röse USR, McAuslane HJ (1996) Systemic induction of feeding deterrents in cotton plants by feeding of *Spodoptera* spp. larvae. J Chem Ecol **22:** 919–932

Baldwin IT, Schultz JC (1983) Rapid changes in leaf chemistry induced by damage: evidence for communication between plants. Science **221:** 277–278

Bowers WS, Nault LR, Webb RE, Dutky SR (1972) Aphid alarm pheromone: isolation, identification, synthesis. Science **177:** 1121–1122

Bruin J, Sabelis MW (1989) Do cotton plants communicate by means of airborn signals? Med Fac Landbouww Rijksuniv Gent **54:** 853–859

Bruin J, Sabelis MW, Dicke M (1995) Do plants tap SOS signals from their infested neighbours? Trees **10:** 167–170

Dicke M, Dijkman H (1992) Induced defence in detached uninfested plant leaves: effects on behavior of herbivores and their predators. Oecologia **91:** 554–560

Dicke M, Sabelis MW (1988) How plants obtain predatory mites as bodyguards. Neth J Zool **38:** 148–165

Dicke M, Sabelis MW, Takabayashi J, Bruin J, Posthumus MA (1990a) Plant strategies of manipulating predator-prey interactions through allelochemicals: prospects for application in pest control. J Chem Ecol **16:** 3091–3118

Dicke M, van Beek TA, Posthumus MA, Ben Dom N, van Bokhoven H, de Groot AE (1990b) Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. J Chem Ecol **16:** 381–396

Elzen GW, Williams HJ, Bell AA, Stipanovic RD, Vinson SB (1985) Quantification of volatile terpenes of glanded and glandless *Gossypium hirsutum* L. cultivars and lines by gas chromatography. J Agric Food Chem **33:** 1079–1082

Gueldner RC, Thomson AC, Hardee DD, Hedin PA (1970) Constituents of the cotton bud. XIX. Attractancy to the boll weevil of terpenoids and related plant constituents. J Econ Entomol **63:** 1819–1821

Heath RR, Manukian A (1992) Development and evaluation of systems to collect volatile semiochemicals from insects and plants using a charcol-infused medium for air purification. J Chem Ecol **18:** 1209–1226

Heath RR, Manukian A (1994) An automated system for use in collecting volatile chemicals released from plants. J Chem Ecol **20:** 593–608

Hollander M, Wolfe DA (1973) Nonparametric Statistical Methods. Wiley & Sons, New York

King EG, Leppla NC (1984) Advances and Challenges in Insect Rearing. U.S. Government Printing Services, Washington, DC

Landolt PJ (1993) Effects of host plant leaf damage on cabbage looper moth attraction and oviposition. Entomol Exp Appl **67:** 79–85

Lewis WJ, Sparks AN, Jones RL, Barras DJ (1972) Efficiency of *Cardiochiles nigriceps* as a parasite of *Heliothis virescens* on cotton. Environ Entomol **1:** 468–471

Loughrin JH, Manukian A, Heath RR, Tumlinson JH (1995) Volatiles emitted by different cotton varieties damaged by feeding beet armyworm larvae. J Chem Ecol **21:** 1217–1227

Loughrin JH, Manukian A, Heath RR, Turlings TCJ, Tumlinson JH (1994) Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plants. Proc Natl Acad Sci USA **91:** 11836–11840

Manukian A, Heath RR (1993) Development of an automated data collection and environmental monitoring system. Sci Comput Autom **9:** 27–40

Mattson WJ Jr (1980) Herbivory in relation to plant nitrogen content. Annu Rev Ecol Syst **11:** 119–161

McCall PJ, Turlings TCJ, Lewis WJ, Tumlinson JH (1993) Role of plant volatiles in host location by the specialist parasitoid *Microplitis croceipes* Cresson (Braconidae: Hymenoptera). J Insect Behav **6:** 625–639

McCall PJ, Turlings TCJ, Loughrin J, Proveaux AT, Tumlinson JH (1994) Herbivore-induced volatile emissions from cotton (*Gossypium hirsutum* L.) seedlings. J Chem Ecol **20:** 3039–3050

Meisner J, Ascher KRS, Zur M (1977) Phagodeterrency induced by pure gossypol and leaf extracts of a cotton strain with high gossypol content in the larva of *Spodoptera littoralis*. J Econ Entomol **70:** 149–150

Nadel H, van Alphen JJM (1987) The role of host- and host-plant odours in the attraction of a parasitoid, *Epidinocarsis lopezi*, to the habitat of its host, the cassava mealybug, *Phenacoccus manihoti*. Entomol Exp Appl **45:** 181–186

Raupp MJ, Denno RF (1983) Leaf age as a predictor of herbivore distribution and abundance. *In* RF Denno, MS McClure, eds, Variable Plants and Herbivores in Natural and Managed Systems. Academic Press, New York, pp 91–124

Sharkey TD, Singsaas EL (1995) Why plants emit isoprene. Nature **374:** 769

Snow JW, Burton RL (1967) Seasonal occurrence of the *Heliothis* complex on *Desmodium purpureum* with observations on parasitism by Cardiochiles nigriceps. J Georgia Entomol Soc **2:** 47–52

Takabayashi J, Dicke M, Posthumus MA (1991) Induction of indirect defence against spider mites in uninfested lima bean leaves. Phytochemistry **30:** 1459–1462

Takabayashi J, Dicke M, Takahashi S, Posthumus MA, Van Beek TA (1994) Leaf age affects composition of herbivore-induced synomones and attraction of predatory mites. J Chem Ecol **20:** 373–386

Takabayashi J, Takahashi S, Dicke M, Posthumus MA (1995) Developmental stage of herbivore *Pseudaletia separata* affects production of herbivore-induced synomone by corn plants. J Chem Ecol **21:** 273–287

Turlings TCJ (1994) The active role of plants in the foraging successes of entomophagous insects. Norw J Agric Sci **16:** 211–219

Turlings TCJ, Loughrin JH, McCall PJ, Röse USR, Lewis WJ, Tumlinson JH (1995) How caterpillar-damaged plants protect themselves by attracting parasitic wasps. Proc Natl Acad Sci USA **92:** 4169–4174

Turlings TCJ, Tumlinson JH (1992) Systemic release of chemical signals by herbivore-injured corn. Proc Natl Acad Sci USA **89:** 8399–8402

Turlings TCJ, Tumlinson JH, Eller FJ, Lewis WJ (1991a) Larval-damaged plants: source of volatile synomones that guide the parasitoid *Cotesia marginiventris* to the micro-habitat of its hosts. Entomol Exp Appl **58:** 75–82

Turlings TCJ, Tumlinson JH, Heath RR, Proveaux AT, Doolittle RE (1991b) Isolation and identification of allelochemicals that attract the larval parasitoid, *Cotesia marginiventris* (Cresson), to the microhabitat of one of its hosts. J Chem Ecol **17:** 2235–2251

Turlings TCJ, Tumlinson JH, Lewis WJ (1990) Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. Science **250:** 1251–1253